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Floating Density of Hepatitis C Virus Particles and Response to Interferon Treatment

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Hepatitis C virus (HCV) particles can be classified into two major fractions according to their floating density in serum. However, the genomic heterogeneity of each fraction and the relationship between this viral characteristic and interferon (IFN) response in patients with chronic hepatitis are not known. In this study, floating density and single strand conformation polymorphism (SSCP) of the hypervariable region 1 (HVR1) of HCV were examined in 16 patients with chronic hepatitis prior to IFN treatment. The ratio of HCV RNA titers in the top (T) and bottom (B) fractions, or T:B ratio, was 10:1 in 4 patients, 1:1 in 7, and 1:10 in 5. Three of the 4 patients with a 10:1 ratio showed a sustained response to IFN, while none of the 5 patients with a 1:10 ratio demonstrated a sustained response ($P < 0.05$). All 4 patients with a 10:1 ratio had 1 or 2 SSCP bands, and 4 of the 5 patients with a 1:10 ratio had 4 or 5 bands ($P < 0.01$). Furthermore, the number of SSCP bands in the top fraction from 6 sustained responders (1.8 ± 0.3) was significantly smaller than from 10 non sustained responders (4.1 ± 0.8) ($P < 0.05$). Thus, patients with a high T:B ratio and low heterogeneity in HVR1 demonstrated sustained responses to IFN, while those with low T:B ratios and high heterogeneity did not. ***J. Med. Virol. 55:12–17, 1998.***

KEY WORDS: single-strand conformation polymorphism; hypervariable region; quasispecies

INTRODUCTION

Interferon (IFN) treatment is the only method available at present with the potential to eliminate hepatitis C virus (HCV) from hepatocytes. However, only 20% of patients with HCV infection treated with IFN eliminate serum HCV RNA [Adrian et al., 1989; Davis et al., 1989; Rumi et al., 1996]. The determinants of IFN response are unknown. Viral factors may influence this response, including the amount of serum HCV RNA [Rumi et al., 1996], the genotype of HCV [Simmonds, 1995], and the presence of interferon sensitivity determining sequences in the HCV genome [Enomoto et al., 1995]. Also host factors including the degree of fibrosis [Tsubota et al., 1994] and the duration of the disease may influence this response.

Genomic heterogeneity of the hypervariable region 1 (HVR1) of HCV may be another viral factor [Koizumi et al., 1995; Moribe et al., 1995]. It has been shown that HVR1 encodes protective epitopes that elicit a specific antibody response [Weiner et al., 1992; Kato et al., 1993; Zibert et al., 1995]. Emergence of new HVR1 variants is followed by the appearance of respective HVR1 specific antibodies [Shimizu et al., 1994], and heterogeneity of HVR1 and the resultant antibodies are found in the serum of patients with chronic hepatitis [Weiner et al., 1992; Kato et al., 1993; Zibert et al., 1995; Nakamoto et al., 1996].

HCV particles have been classified into two major populations according to their floating density, with the low density particles have been reported to have higher infectivity than the high-density particles [Hijikata et al., 1993; Kanto et al., 1994]. The density of the former particles is close to that of low-density lipoprotein, and the majority of particles are coprecipitate with anti-β lipoprotein [Thomssen et al., 1992]. The high density particles can be precipitated with anti-immunoglobulin, and are thought to be present as heterogeneous antigen-antibody complexes in serum [Thomssen et al., 1993]. These findings suggest that a heterogeneous population of HCV and antibodies may be present in the high-density population. However, the relationship between the genomic heterogeneity of HVR1 and its floating density is not known. The ratio of the two populations is known to change during chronic infection or IFN treatment in each individual, and may vary among patients with chronic hepatitis [Kanto et al., 1995a; Nagasaka et al., 1996]. The density of HCV and the genomic heterogeneity of each density population was examined in the serum of patients

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TABLE I. Clinical Characteristics of Patients with Chronic Hepatitis C

Patient number	Age	Sex	Blood transfusion (age at transfusion)	HCV RNA titer[a]	Effect of IFN
1	37	M	+ (30)	10^5	SR
2	34	M	–	10^5	SR
3	55	F	+ (50)	10^6	SR
4	46	M	+ (23)	10^7	SR
5	56	F	–	10^7	SR
6	45	M	+ (12)	10^7	SR
7	50	M	+ (42)	10^4	NR
8	32	M	–	10^4	NR
9	63	M	–	10^5	NR
10	62	M	–	10^6	NR
11	53	F	–	10^6	NR
12	45	M	–	10^6	NR
13	63	F	+ (40)	10^7	NR
14	50	M	–	10^7	NR
15	50	M	–	10^7	NR
16	52	M	+ (42)	10^7	NR

Abbreviations: SR, sustained responder; NR, nonsustained responder.
[a]HCV RNA titer; copies per milliliter of serum.

with chronic hepatitis, in an attempt to clarify the relationship of this viral character to IFN response.

MATERIALS AND METHODS

Patients

Sixteen patients with chronic hepatitis C admitted to Kanazawa University Hospital between March 1992 and June 1993 were studied (Table I). All 16 patients were seropositive for antibodies to HCV (anti-HCV) and HCV RNA, and were infected with genotype 1b. None of the patients were homosexual, intravenous drug users, or positive for either hepatitis B virus surface antigen or antinuclear antibodies. All patients underwent liver biopsy, and a histologic diagnosis was made using accepted criteria [International group, 1977]. Informed consent was obtained from all patients before IFN treatment. All patients received recombinant IFN α 2a at a dose of 6 MU/day for 2 weeks, and 3 times a week for 22 weeks. A sustained response was defined as the normalization of alanine transaminase (ALT) within 6 months of the initiation of treatment and its continuation over 6 months after the completion of treatment. Six patients demonstrated a sustained response (sustained responders).

Differential Flotation Centrifugation

Differential flotation centrifugation was carried out according to the method of Havel et al. [1955]. Six hundred μL of each serum sample collected before IFN treatment was layered on top of 4,400 μL of an NaCl solution (density 1.063 g/mL). The mixture was centrifuged at 139,500 × *g* (37,000 rpm) for 22 hr at 14°C in a Beckman 55 Ti rotor (Beckman Instruments, Palo Alto, CA, USA). After centrifugation, the top and bottom fractions (400 μL each) were collected, and were stored at –20°C.

Quantification of HCV RNA Titer in Serum and Each Fraction

To quantify the HCV RNA titer in serum, HCV RNA was measured in a 10-fold serial dilution of 10 μL serum using a single-tube polymerase chain reaction (PCR) assay [Hayashi et al., 1994]. The standard serum sample, containing 10^7 copies/mL of strain H [Feinstone et al., 1981; Hijikata et al., 1993; Hayashi et al., 1994], was positive at a dilution of 10^4 but not at 10^5, indicating the need for more than 10^3 copies/mL HCV (10^1 copies/10 μL) in serum. The amount of HCV RNA in the top and bottom fractions was measured, and the ratio was expressed as the T:B ratio. All serum and fraction samples were extracted and analyzed in parallel with standard serum samples and negative controls.

SSCP Analysis

Heterogeneity of HVR1 was analyzed by a PCR-based single strand conformation polymorphism (SSCP) method [Orita et al., 1989] using HVR1 primers [Enomoto et al., 1994]. After extraction of RNA from 100 μL of sample, cDNA was synthesized with random hexamers (Takara, Kyoto, Japan) using Molony murine leukemia virus reverse transcriptase (SuperScript™ II RNase H$^-$, Gibco BRL, Gaithersburg, MD). The cDNA was mixed with each outer primer and *Taq* polymerase (Ex Taq, Takara), and PCR amplified. Single stranded cDNA was amplified by nested asymmetric PCR using either 5′- or 3′-inner primers. PCR products were subjected to 5% nondenaturing PAGE. The gel was stained with ethdium bromide, and DNA was visualized under UV light.

Oligonucleotide Primers

The oligonucleotide primers used to amplify HCV by PCR were synthesized with a 390 DNA Synthesizer (Applied Biosystems, Foster City, CA, USA) based on published nucleotide sequences. For quantification of HCV RNA, we used 5′ noncoding (5′NC) region primers. The outer primers were designated 23 (5′-ACTCCACCATAGATCACTCC-3′ [sense]) and 351R (5′-TTGTGCTCATGGTGCACG-3′ [antisense]). The

TABLE II. T:B Ratio, SSCP Analysis and Effect of IFN Therapy

Patient number	HCV RNA titer[a]	Effect of IFN	T:B ratio	Number of SSCP bands		
				Serum	Top	Bottom
1	10^5	SR	1:1	2	2	2
2	10^5	SR	10:1	2	2	5
3	10^6	SR	1:1	1	1	2
4	10^7	SR	10:1	1	2	2
5	10^7	SR	10:1	1	1	2
6	10^7	SR	1:1	2	3	4
7	10^4	NR	1:1	2	6	4
8	10^4	NR	10:1	2	2	2
9	10^5	NR	1:1	4	3	3
10	10^6	NR	1:10	4	3	3
11	10^6	NR	1:10	4	3	5
12	10^6	NR	1:10	5	4	5
13	10^7	NR	1:1	3	2	2
14	10^7	NR	1:1	3	5	3
15	10^7	NR	1:10	4	3	3
16	10^7	NR	1:10	2	10	6

Abbreviations: SR, sustained responder; NR, nonsustained responder.
[a]HCV RNA titer; copies per milliliter of serum.

corresponding inner primers were designated 37 (5′-CACTCCCCTGTGAGCTACTG -3′ [sense]) and 346R (5′-CTCATGGTGCACGGTCTACGAGACC-3′ [antisense]) [Hayashi et al., 1994]. For SSCP analysis, we used primer pairs to detect 351 bases of hypervariable region 1 of the virus. The outer primers were designated 1261 (5′-GCCATTTATCAGGTCACCGCATGGC-3′ [sense]) and 1692R (5′-GCTCCGGGCACCCGGAC-GAGTTGAA-3′ [antisense]). The corresponding inner primers were designated 1284 (5′-GCTTGGGATAT-GATGATGAACTGGTC-3′ [sense]) and 1634R (5′-GGTGTGGAGGGAGTCATTGCAGTT [antisense]) [Enomoto et al., 1994].

Statistical Analysis

Fisher's exact probability test or Student's *t*-test was used for statistical analysis of differences between frequencies in the two groups. Results are expressed as mean ± SEM. A P value of <0.05 was considered to be significant.

RESULTS

Amount of HCV RNA in Top and Bottom Fractions

Serum HCV RNA was fractioned into two fractions (Top, T; and Bottom, B) according to their floating density. The amount of HCV RNA in each fraction was measured, and the ratio of HCV RNA levels was expressed as the T:B ratio (Table II). All patients had HCV RNA in both fractions irrespective of the amount of whole serum HCV RNA or IFN response. Four patients had a 10:1 T:B ratio of serum HCV RNA, 7 had 1:1, and 5 had 1:10 before IFN treatment. Among 5 patients with less than 10^5 copies/mL of serum HCV RNA, 2 had a 10:1 T:B ratio, and 3 had 1:1 (Fig. 1). Two of 11 patients with larger than 10^6 copies/mL of serum HCV RNA had a 10:1 ratio, 4 had 1:1, and 5 had 1:10. Thus, the T:B ratio decreased with an increase in the total amount of HCV RNA, but the difference was not statistically significant.

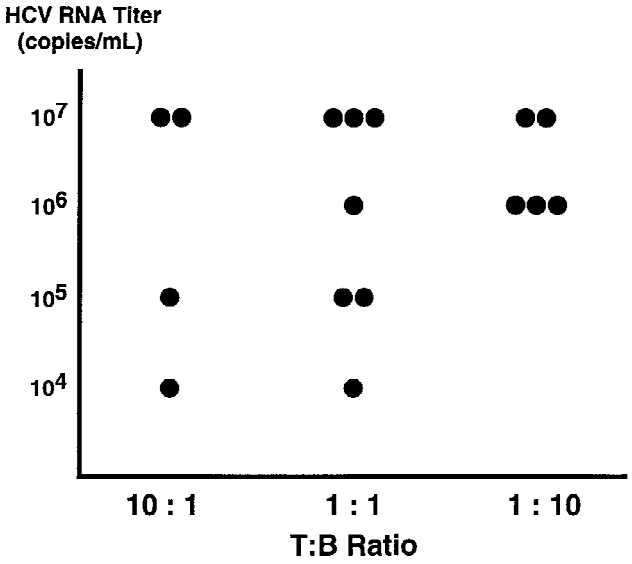

Fig. 1. T:B ratio and titer of HCV RNA in serum. The titer of serum HCV RNA was measured by PCR using a 10-fold serial dilution of 10 μL of serum. The titer of HCV RNA in the top and bottom fractions was measured, and the ratio of top and bottom fractions was expressed as the T:B ratio.

The T:B ratio to IFN response was also compared (Table III). Three of 4 patients (75%) who had a 10:1 T:B ratio of serum HCV RNA demonstrated a sustained response. On the other hand, all 5 patients (100%) who had a 1:10 ratio did not respond to IFN therapy. Thus, patients with a 10:1 T:B ratio significantly responded to IFN therapy compared with those with a 1:10 ratio ($p<0.05$). Among 7 patients with 10^7 copies/mL HCV RNA, both patients with a 10:1 ratio of HCV RNA and 1 of 3 patients with 1:1 responded to IFN therapy.

TABLE III. T:B Ratio and Effect of IFN Therapy

T:B ratio			Effect of IFN		
Top	Bottom	Total	SR	NR	
10:1		4	3 (75%)	1 (25%)	[a]
1:1		7	3 (44%)	4 (56%)	
1:10		5	0 (0%)	5 (100%)	

Abbreviations: SR, sustained responder; NR, nonsustained responder.
[a] $P < 0.05$.

Genomic Heterogeneity of HVR1 in Each Fraction

The genomic heterogeneity of HCV in each fraction was analyzed by SSCP using HVR1 primers. Results from patient no. 12, who had 10^6 copies/mL of serum HCV RNA and a 1:10 T:B ratio, are shown in Fig. 2. Bands were visualized in each lane, and only clear bands were counted as SSCP bands. Similarly, genomic heterogeneity of HVR1 was found in each fraction as well as whole serum, and 1 to 10 clear bands were demonstrated in each sample (Table II). The number of bands from whole serum samples was similar to that in the dominant HCV RNA fraction (top or bottom fraction), except for patient nos. 7 and 16. However, the size of each band in serum samples did not always correspond to that found in each fraction.

There was no relationship between the amount of HCV RNA and the number of SSCP bands in whole serum or individual fractions (Fig. 3). All four patients with a 10:1 T:B ratio had one or two SSCP bands in serum, while four of five patients with a 1:10 T:B ratio had four or five bands. Similarly, when the T:B ratio decreased, the number of SSCP bands in the top or bottom fractions increased, indicating that the relative amount of HCV in the bottom fraction correlates with genomic heterogeneity (Fig. 4). The relationship between the number of bands in each fraction and IFN response was also studied (Fig. 5). The number of SSCP bands in whole serum from 6 sustained responders (1.5 ± 0.2) was significantly smaller than that from 10 nonsustained responders (3.3 ± 0.3) ($P < 0.01$). Similarly, the number of bands in the top fractions from sustained responders (1.8 ± 0.3) was significantly smaller than that from nonsustained responders (4.1 ± 0.8) ($P < 0.05$). Thus, genomic heterogeneity in whole serum and top fractions was closely related to IFN response. Patients with high T:B ratios and low heterogeneity in HVR1 demonstrated sustained response to IFN irrespective of high titers of serum HCV RNA, while those with low T:B ratios and high heterogeneity did not.

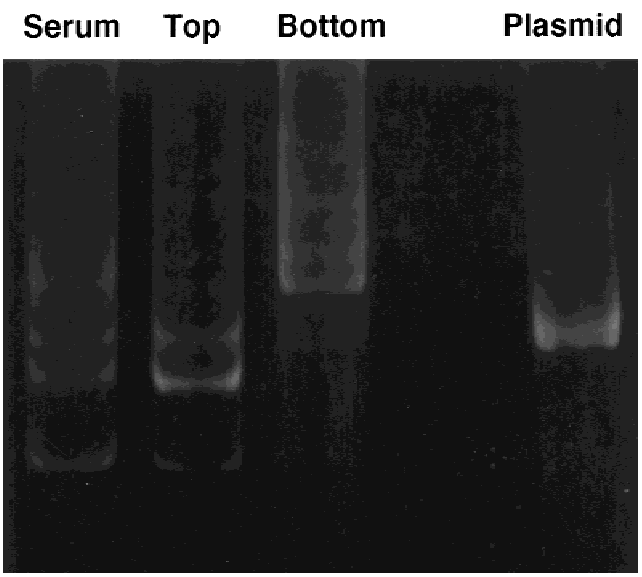

Fig. 2. Single strand conformation polymorphism (SSCP) analysis of HCV in patient 12. Heterogeneity of hypervariable region 1 (HVR1) of HCV in serum, top and bottom fractions was analyzed by PCR-mediated SSCP using HVR1 primers. A plasmid HCV-JK1 (EMBL accession no. X61591) was used as a single population control. The PCR product was subjected to nondenaturing PAGE, electrophoresed, and distinct bands were counted as SSCP bands.

DISCUSSION

HCV particles in the serum of 16 patients with chronic hepatitis were separated into two fractions, top and bottom, according to their floating density [Hijikata et al., 1993; Kanto et al., 1994]. The ratio of HCV RNA in the two fractions (T:B ratio) was different in each patient, and ranged from 10:1 to 1:10. This ratio has been reported to change in each individual during the course of disease. In acute hepatitis, HCV in the top fraction dominated the early phase of infection both in chimpanzees and humans, with virus subsequently moving from the top to bottom fraction in the late phase [Hijikata et al., 1993]. HCV particles in the top fraction also decreased soon after IFN treatment in chronic hepatitis, and the T:B ratio increased before a flare-up of serum ALT in chronic hepatitis [Kanto et al., 1995b; Nagasaka et al., 1996]. These findings imply that this viral characteristic may correlate with IFN response. In fact, Nagasaka et al. showed that all four patients with a 1:1 T:B ratio of HCV RNA partially responded to IFN therapy, while six of eight patients with a 1:10 T:B ratio did not respond, although no complete responders were studied [Nagasaka et al., 1996]. In our study, three of four patients with a 10:1 T:B ratio demonstrated a sustained response to IFN, while all five patients with a 1:10 T:B ratio did not. Thus, patients with a high T:B ratio of HCV particles may respond to IFN better than those with a low T:B ratio.

The majority of HCV particles in the top fraction are coprecipitate with anti-β lipoprotein but not with anti-immunoglobulin [Thomssen et al., 1993]. The bottom particles precipitate with anti-immunoglobulin, and are considered to be present as an antigen-antibody complexes in serum [Hijikata et al., 1993; Kanto et al., 1995]. Although the character of the complex is not fully understood, HVR1 within the E2 protein of HCV is known to contain isolate-specific antibody-binding epitopes, and a heterogeneous population of HVR1 and antibodies against HVR1 can be found in the serum of patients with chronic hepatitis [Weiner et al., 1992;

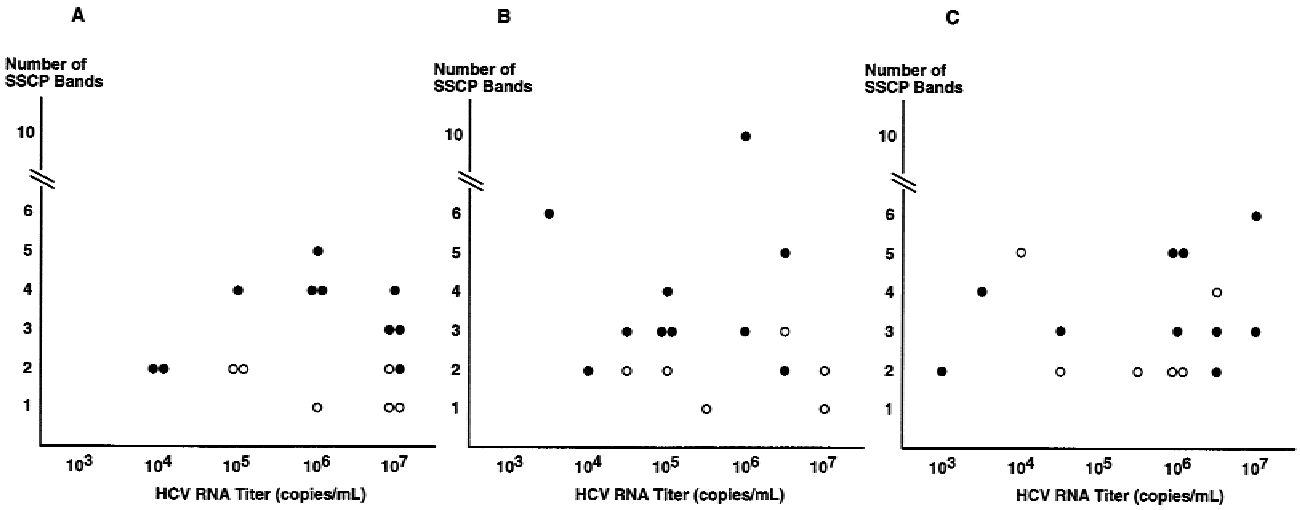

Fig. 3. Titer of HCV RNA and number of SSCP bands in serum (A), top (B) and bottom (C) fractions. There were 6 sustained responders (SR) and 10 nonresponders (NR) to interferon. Open and closed circles indicate sustained responders and nonsustained responders, respectively.

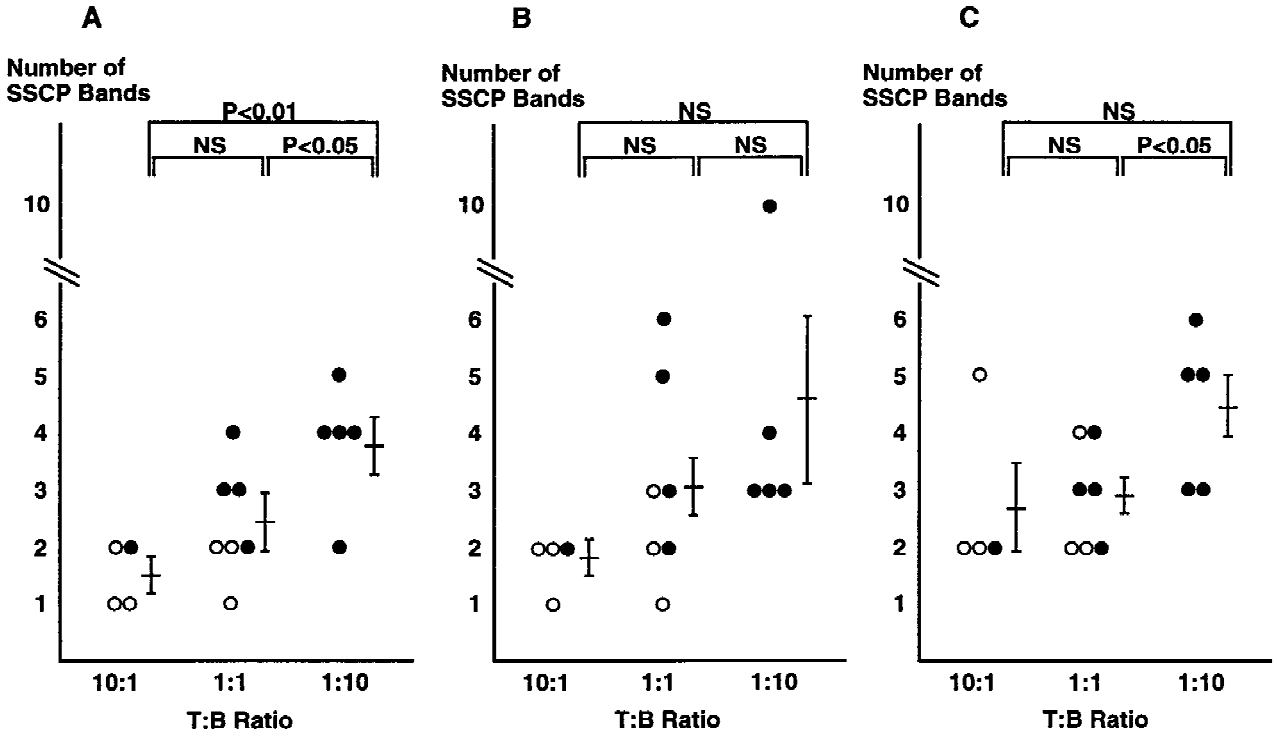

Fig. 4. T:B ratio and number of SSCP bands in serum (A), top (B) and bottom (C) fractions. Open and closed circles indicate sustained responders and nonsustained responders to interferon, respectively.

Kato et al., 1993; Zibert et al., 1995; Nakamoto et al., 1996]. These findings suggest that HCV newly released from hepatocytes is free of antibody-reacting epitopes (top fraction), but heterogeneous HCV populations with reacting epitopes, make a complex with antibodies (bottom fraction). To determine whether genomic heterogeneity of HVR1 is present in the bottom fraction and correlates with IFN response [Koizumi et al., 1995; Moribe et al., 1995], SSCP of serum and each fraction was performed.

The SSCP assay demonstrated the presence of genomic heterogeneity of HVR1 not only in whole serum and the bottom fraction but also in the top fraction. Similar to previous studies [Koizumi et al., 1995; Moribe et al., 1995], the number of SSCP bands in whole serum from sustained responders was significantly smaller than that of nonresponders. This finding was also demonstrated in the top fractions, however, in bottom fractions differences were not statistically significant. When HCV in the top fraction was dominant

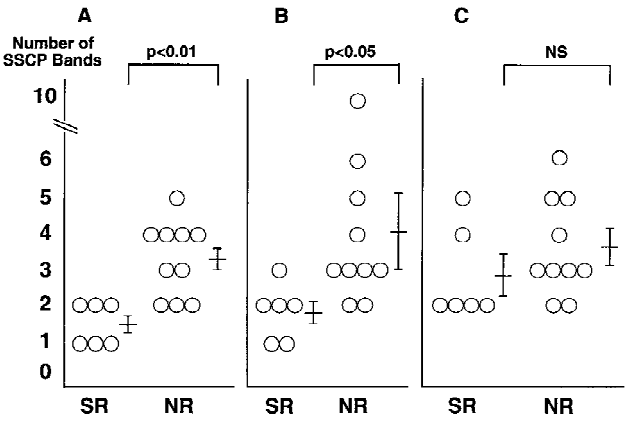

Fig. 5. Interferon response and number of SSCP bands in serum (A), top (B) and bottom (C) fractions. There was a statistically significant difference between sustained responders (SR) and nonsustained responders (NR) in serum ($P < 0.01$) and top fractions ($P < 0.05$), respectively.

(i.e. a 10:1 T:B ratio), HVR1 was less heterogeneous in serum and both fractions, and a sustained response to IFN was seen (Fig. 4). On the other hand, when HCV in the bottom fraction was dominant (i.e. a 1:10 ratio), HVR1 was more heterogeneous, and a sustained response was not present. All patients were infected with genotype 1b of HCV, had chronic active hepatitis and received the same IFN protocol in this study. However, several factors influencing the effect of IFN, such as amount of HCV RNA and interferon sensitivity, need to be evaluated independently, and further study is clearly necessary to establish that floating density of HCV is one of the viral traits determining IFN response.

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